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Rat fibroblasts synthesize T-kininogen in response to cyclic-AMP, prostaglandin E_2 and cytokines

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Abstract

T-Kininogen is a plasma protein characterized as a kinin-precursor, a cysteine protease inhibitor and an acute phase protein in the rat. Rat fibroblasts prepared from meninges or embryos and 3Y1-B clone 1-6 cells, a rat fibroblast cell line, secreted T-kininogen. Incubating these cells with 1 mM Bt_2cAMP or a combination with 1 μM dexamethasone resulted in a marked increase in T-kininogen secretion, as well as in the incorporation of radioactive methionine into newly synthesized T-kininogen. Secretion of T-kininogen by meningeal fibroblasts was stimulated by forskolin, prostaglandin E_2, bradykinin and cytokines, such as tumor necrosis factor α, interleukin-1α (IL-1) and IL-6. Expression of T-kininogen mRNA was demonstrated in meningeal fibroblasts by Northern blot hybridization using T-kininogen cDNA as a probe, and the expression was stimulated by Bt_2cAMP, prostaglandin E_2, and the cytokines described above. In contrast, expression of T-kininogen mRNA in rat hepatocytes was not altered by Bt_2cAMP, prostaglandin E_2, tumor necrosis factor and IL-1, whereas it was greatly stimulated by IL-6, suggesting the differential regulation of T-kininogen gene expression in fibroblasts and hepatocytes. These results demonstrated for the first time, that rat fibroblasts express the T-kininogen gene, and that the expression is regulated by inflammatory mediators and cytokines.

Keywords: Kininogen; T-kininogen; Fibroblast; cyclic AMP; Prostaglandin E_2; Cytokine

1. Introduction

Kininogens are endogenous protein substrates for kallikreins, which by proteolytic cleavage form vasoactive kinin peptides [1]. In addition, kininogens contain domains that function as cysteine proteinase inhibitors and act as cofactors for contact activation of blood coagulation [1]. Three forms of kininogens have so far been found in mammalian plasma: high molecular weight kininogen (H-kininogen), low molecular weight kininogen (L-kininogen), and T-kininogen. The latter is unique to the rat [2]. Although T-kininogen is highly homologous in structure with L-kininogen and releases a unique bradykinin analogue T-kinin (isoleucyl-seryl-bradykinin) upon exposure to trypsin [3], it is resistant to cleavage by kallikreins [4]. In addition, T-kininogen is recognized as an acute phase protein, whose levels in the circulation increase several-fold during inflammation [5,6].

The kallikrein-kinin system is considered to be a biochemical cascade in plasma and other body fluids whose final products, kinins, are potent vasodilators. They are assumed to be involved in various diseases including inflammation [7]. In the circulation, all components of the kallikrein-kinin system are present, i.e., plasma kallikrein as a form of zymogen, tissue kallikrein, and their substrates H- and L-kininogen [8]. In local tissues and organs, tissue kallikrein, by which lysyl-bradykinin is released from L-kininogen, is ubiquitously distributed in the kidney, blood vessels, brain, pituitary, pancreas, intestine, spleen and adrenal glands [9]. Although kininogens in the circulation have been considered the source of kallikrein substrate in local tissues, recent studies in humans have demonstrated that L- and H-kininogen genes are expressed in the kidney and endothelial cells, respectively [10,11], suggesting the existence of a kallikrein-kinin system in the local tissues that is independent of the circulating counter-

Abbreviations: H-kininogen, high-molecular-weight kininogen; L-kininogen, low-molecular-weight kininogen; Bt_2cAMP, dibutyryl cAMP; PGE_2, prostaglandin E_2; TNF, tumor necrosis factor α; IL-1, interleukin-1α; IL-6, interleukin-6; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium.

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part. In the rat, the immunohistochemical techniques demonstrate localizations of H-and/or T-kininogens in pituitary, heart, lung, kidney, thymus, spinal cord, hypothalamus and brain stem [12,13]. T-Kininogen-like immunoreactivity is also detected in rat brain tissues and cells [14], suggesting the de novo synthesis of T-kininogen by rat brain. In addition, low levels of T-kininogen gene expression have recently been identified in the rat in extrahepatic tissues including the lung, kidney, brain, and heart [15], though it is unknown which types of cells in these tissues express T-kininogen gene. These lines of evidence led us to seek cells which could synthesize and secrete T-kininogen in vitro. Here, we demonstrated for the first time that rat fibroblasts synthesized and secreted T-kininogen in vitro in response to inflammatory mediators and cytokines.

2. Materials and methods

2.1. Materials

The following chemicals were obtained from commercial sources: Bt_2cAMP from Boehringer-Mannheim-Yamanouchi; forskolin, trypsin (bovine pancreas) and protein A-agarose from Sigma; horseradish peroxidase-labeled avidin and biotinylated goat anti-rabbit IgG from Bio-Rad; bradykinin, T-kinin and leupeptin from the Peptide Institute (Osaka, Japan); Na[^{125}I] (94.3 GBq/μmol) and [α-^{32}P]dCTP (37 GBq/μmol) from American Radiolabeled Chemicals; Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium (MEM) from Nissui (Osaka, Japan); Williams' medium E from ICN Biomedicals; rabbit anti-rat fibronectin from Funakoshi (Tokyo, Japan); rabbit anti-human von Willebrand factor from Dako Japan; rabbit anti-human glial fibrillary acid protein from Upshaw; octadecyl disposable column from J.T. Baker; 3Y1-B 1-6 cells (3Y1 cells) from Riken Cell Bank (Tsukuba, Japan). Recombinant human interleukin-1α (IL-1; $2 \cdot 10^7$ U/mg) and tumor necrosis factor α (TNF; $3 \cdot 10^6$ U/mg) were donated by Dainippon Pharmaceuticals (Osaka, Japan). Prostaglandin E_2 (PGE_2) was a gift from Ono Pharmaceuticals (Osaka, Japan). Recombinant human interleukin-6 (IL-6; $5.2 \cdot 10^6$ U/mg) was supplied by Dr. T. Hirano (Osaka University Medical School). T-Kininogen was purified from rat plasma as described [2].

2.2. Cell culture

Fibroblasts were prepared from the meninges and the embryos of Sprague–Dawley rats as follows. The meninges was isolated from hemispheres of 1-day-old newborn rats, then digested with 0.05% trypsin/0.02% EDTA for 2 min at 37° C. The meningeal cells obtained were washed and cultured in DMEM containing 5% fetal bovine serum (FBS) and 50 μg/ml of gentamycin (growth medium) at 37° C in a humidified atmosphere of 95% air and 5% CO_2. Rat embryonic fibroblast cultures were initiated from 16- to 18-day decapitated and degutted embryos. The embryonic skin was washed with phosphate-buffered saline (pH 7.5), cut with scissors, then digested twice by stirring gently in 0.05% trypsin/0.02% EDTA for 15 min each. The filtrate that passed through a stainless steel net (mesh size of 150 μm) was diluted in growth medium, then seeded in 90-mm culture dishes in the same medium.

After the meningeal and embryonic fibroblasts reached confluence, they were passaged by disruption with trypsin and reseeded onto 24-well culture plates or 90-mm culture dishes. The cells that reached confluence again were used for experiments. These cultures contained mainly fibronectin-positive cells (> 95%), when tested with rabbit antiserum directed against rat fibronectin. The cultured meningeal cells were negative to staining with either a rabbit antibody against glial fibrillary acidic protein, a marker protein of astrocytes, or a rabbit antiserum directed against von Willebrand factor, a marker for endothelial cells. When studying the effect of dexamethasone on these cells, the FBS in the medium was adsorbed with activated charcoal to lower endogenous glucocorticoids levels [16] was used.

An established line of rat fibroblasts, 3Y1, derived from an embryo [17] was maintained in the growth medium as described above.

Parenchymal hepatocytes were isolated by in situ perfusion of livers with collagenase from male Sprague–Dawley rats, weighing 120–130 g, essentially as described [18]. The cells ($1.8 \cdot 10^5$) were plated in 24-well culture plates in 1 ml of Williams' E medium supplemented with 5% FBS, 10 nM insulin, 10 nM dexamethasone and 50 μg/ml of gentamycin, and cultured at 37° C in 5% CO_2–95% air. The medium was replaced after the first 2 h of culture to remove unattached cells. After a further 24 h, cells were used for study.

2.3. Assay of T-kininogen

T-Kininogen in culture medium was determined by a radioimmunoassay using ^{125}I-T-kininogen and rabbit anti-T-kininogen antibody, as described previously [19]. This assay detected 2 ng T-kininogen/ml. The intra-assay precision, as represented by the coefficient of variation of duplicates ranging throughout the standard curve, was $\pm$5.1%, and the inter-assay reproducibility was 6.7%. Results were expressed as the means $\pm$ S.D. of the amount of T-kininogen/mg protein of cells. Cell protein was measured using the BCA Protein Assay Reagent (Pierce).

2.4. Western blots of T-kininogen

Lyophilized fibroblast conditioned medium was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gel according to the

method of Laemmli [20]. The resolved proteins were transferred to a nitrocellulose membrane, which was then blocked with 10% skim milk in 20 mM Tris-HCl (pH 7.5) for 1 h, and washed with the buffer. The blot was then incubated with 200-fold-diluted rabbit anti-T-kininogen serum for 2 h at room temperature. The reaction products were developed by the avidin-biotin-peroxidase complex method according to the instruction manual issued by Bio-Rad Laboratories.

2.5. Radiolabeling of fibroblast cultures and immunoprecipitations

Fibroblasts were grown to confluency in 24-well culture plates and washed with FBS-free DMEM, and the medium was replaced with 0.5 ml of growth medium containing the test agents, 1 mM Bt_2cAMP and 1 μM dexamethasone. After 72 h, the cultures were rinsed three times with methionine-deficient MEM, and 0.5 ml of methionine-deficient MEM supplemented with [^{35}S]methionine (3.7 MBq/ml) was added. The cultures were incubated for 24 h, and the medium was harvested and centrifuged at $8000 \times g$. The labeled supernatant was combined with an equal volume of 20 mM Tris-HCl buffer (pH 7.4) containing 0.28 M NaCl, 5 mM EDTA, 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml of leupeptin and 0.2% bovine serum albumin. An aliquot of the treated labeled culture medium (50 μl) was mixed with 0.5 ml of immunoprecipitation buffer (I-buffer; 10 mM Tris-HCl [pH 7.4] containing 2.5 mM EDTA, 0.14 M NaCl and 0.5% Triton X-100) and 0.5 μl of rabbit anti-T-kininogen serum. In some immunoprecipitation tubes, 20 μg/ml of rat T-kininogen was added to compete with radiolabeled T-kininogen for antibody binding. After an incubation at 4° C for 18 h, 100 μl of a 20% suspension of Protein A-agarose was added, mixed and incubated for 2 h at 4° C. The gel was washed five times with I-buffer, resuspended in 100 μl of 0.125 M Tris-HCl (pH 6.8) containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.2% bromophenol blue, then heated in boiling water for 2 min. The supernatant (20 μl) was loaded onto a 10% polyacrylamide gel containing 0.1% SDS and resolved by electrophoresis. The gel was then fluorographed using sodium salicylate [21], then dried and autoradiographed with X-ray films at −90° C.

2.6. RNA extraction and Northern blots

The plasmid prKG-51 containing rat cDNA encoding T-kininogen [22] was supplied by Dr. N. Kitamura (Kansai Medical University, Osaka) and a 702-base pair *Sau*3A1 fragment was the hybridization probe. The cDNA fragment was radiolabeled with [α-^{32}P]dCTP by random priming [23]. Total RNA was isolated from meningeal fibroblasts and hepatocytes by the method of Chomczynski and Sacchi [24]. Aliquots (20 μg for fibroblasts and 10 μg for hepatocytes) of total RNA were electrophoresed in a 1.2% agarose gel containing 20 mM 3-(N-morpholino)propanesulfonic acid, 1 mM EDTA, 8 mM sodium acetate (pH 4.0), and 2.2 M formamide and transferred onto a nylon membrane (Hybond N+; Amersham). The membranes were irradiated with UV (4.5 J/cm^2) and prehybridized at 65° C for 30 min in hybridization buffer (1% bovine serum albumin, 1 mM EDTA, 7% SDS and 5% formamide in 0.5 M sodium phosphate [pH 7.2]) [25], following which they were hybridized in hybridization buffer at 65° C with [α-^{32}P]cDNA for 18 h, and rinsed with 1 mM EDTA and 1% SDS. The blots were autoradiographed and evaluated using a Fuji Film Bio Imaging Analyzer BAS1000.

2.7. Statistical analysis

Results are presented as mean ± S.D. Statistics between sample means were initially compared using the one-way analysis of variance (ANOVA), then if this proved significant, the differences were tested by Bonferroni's method.

3. Results

3.1. Secretion of T-kininogen by rat fibroblasts

We first investigated whether the culture medium of rat meningeal fibroblasts contained material that reacted with a rabbit anti-T-kininogen antibody. The meningeal fibroblasts were cultured for 4 days, then T-kininogen in the conditioned medium was radioimmunoassayed. T-kininogen-like immunoreactivity was detected at a level of 85 ± 40 ng/mg cell protein ($n = 3$). Western blotting using rabbit anti-T-kininogen antibody demonstrated that the conditioned medium contained a virtually single immunoreactive band that was indistinguishable from rat plasma T-kininogen corresponding to a molecular mass of 68 kDa (Fig. 1). When the conditioned medium of meningeal fibroblasts was incubated with trypsin, and then generated kinin peptides were separated by high performance liquid chromatography, kinin radioimmunoassay using anti-bradykinin antibody identified T-kinin (data not shown). These results indicated that meningeal fibroblasts secreted T-kininogen.

3.2. Secretion of T-kininogen by fibroblasts in response to Bt_2cAMP

When meningeal fibroblasts were cultured in DMEM medium containing FBS for 4 days in the presence of 1 mM Bt_2cAMP, T-kininogen dramatically increased from the basal levels of 75 ± 15 ng/mg cell protein to 2330 ± 424 ng/mg (Fig. 2). The secretion of T-kininogen by meningeal fibroblasts was further increased by a combination of 1 mM Bt_2cAMP and 1 μM dexamethasone, though dexamethasone alone did not alter T-kininogen secretion,

indicating that dexamethasone synergistically enhanced the Bt_2cAMP-induced increase in T-kininogen production (Fig. 2). Rat embryonic fibroblasts also secreted T-kininogen, and the secretion was increased only 2-fold by 1 mM Bt_2cAMP alone, but was further enhanced more than 10-fold by a combination of 1 mM Bt_2cAMP and 1 μM dexamethasone (Fig. 2). We also found that the rat fibroblast cell line 3Y1 secreted T-kininogen, which was increased about 5-fold by 1 mM Bt_2cAMP and 1 μM dexamethasone, whereas neither Bt_2cAMP nor dexamethasone alone had any effect (data not shown).

In contrast to fibroblasts, rat hepatocytes in primary cultures secreted more T-kininogen (22.8 μg/mg cell protein in 2 days of culture), and the addition of 1 mM Bt_2cAMP to the cultures had no significant effect on T-kininogen secretion (data not shown).

To investigate whether Bt_2cAMP or dexamethasone influenced the de novo synthesis of T-kininogen, fibroblasts, which had been pretreated with or without 1 mM

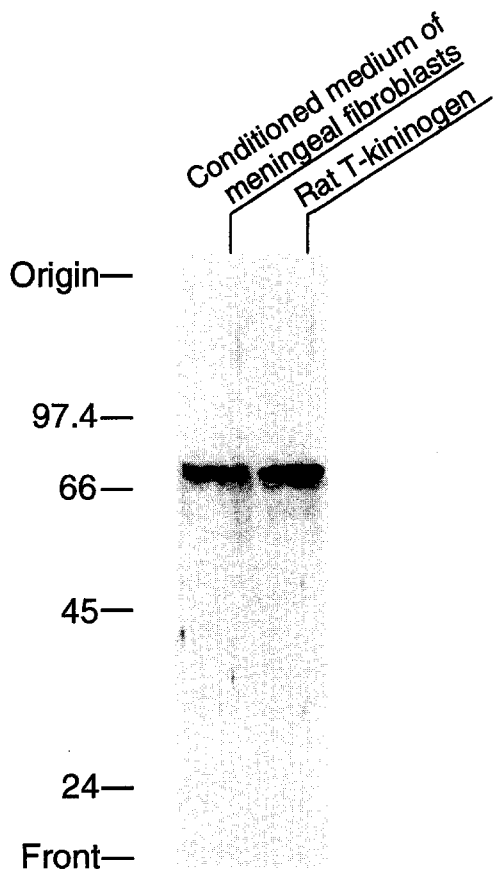

Fig. 1. Western blots of T-kininogen-like immunoreactive material in the conditioned medium of rat meningeal fibroblasts. Meningeal fibroblasts were cultured for 4 days, then the conditioned medium was harvested, dialyzed against distilled water and lyophilized. The lyophilized sample (left) and rat plasma T-kininogen (right) were resolved by SDS-PAGE in a 10% gel and transferred to nitrocellulose. The blot was incubated with anti-T-kininogen antibody followed by goat biotinylated anti-rabbit IgG, then with horseradish-labeled avidin. The numbers to the left of the blot represent molecular mass standards in kDa.

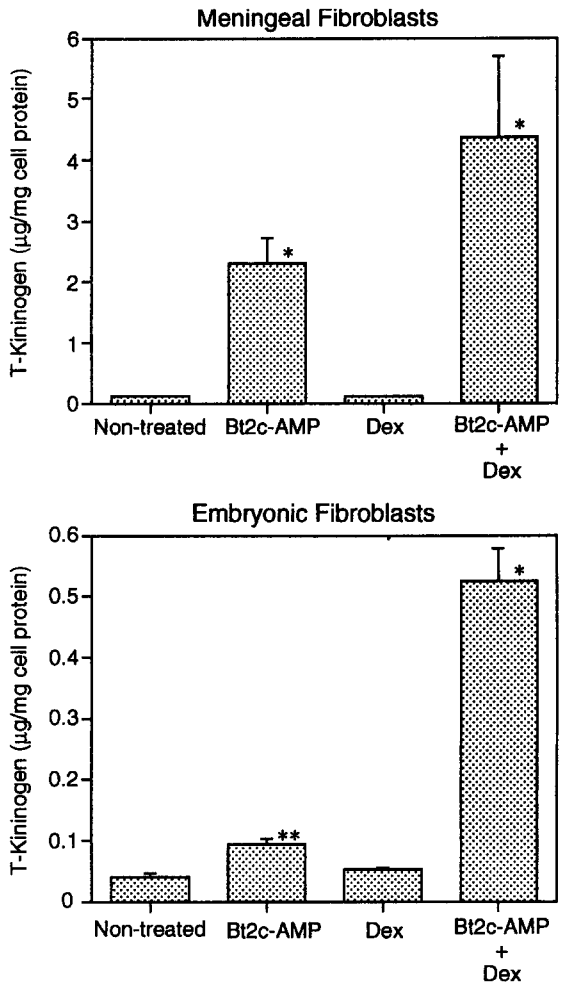

Fig. 2. The effects of Bt_2cAMP and dexamethasone on the secretion of T-kininogen by rat fibroblasts. Rat fibroblasts from meninges (upper panel) or embryos (lower panel) were cultured in 24-well plates with or without Bt_2cAMP (1 mM) and/or dexamethasone (1 μM) for 4 days, and the T-kininogen in the medium was assayed by T-kininogen radioimmunoassay. Untreated meningeal or embryonic fibroblasts secreted 75 ± 15 ng or 42 ± 4 ng/mg cell protein for 4 days culture, respectively. The results are expressed as the means ± S.D. of 4 wells. * Statistical analysis in comparison to unstimulated culture ($P < 0.001$).

Bt_2cAMP and/or 1 μM dexamethasone, were metabolically labeled with [^{35}S]methionine, then the conditioned medium was immunoprecipitated using anti-T-kininogen antibody (Fig. 3). Although there was no detectable band corresponding to T-kininogen in immunoprecipitates from the conditioned medium of meningeal fibroblasts cultured without Bt_2cAMP (lanes 2 and 4 in Fig. 3), incubating the cells with Bt_2cAMP resulted in the appearance of a metabolically labeled protein that migrated with the molecular mass of ^{125}I-T-kininogen (lanes 3 and 5). When immunoprecipitated in the presence of the excess amount of no radiolabeled T-kininogen, the radioactive band corresponding to T-kininogen disappeared (lane 6). Similar results were obtained in immunoprecipitates from the culture medium of metabolically labeled embryonic fibroblasts (Fig. 3). A radioactive band corresponding to T-

kininogen was also observed in the conditioned medium of 3Y1 cells which had been incubated with both Bt_2cAMP and dexamethasone, but not in those incubated with Bt_2cAMP alone (Fig. 3). These results demonstrate the de novo synthesis of T-kininogen by rat fibroblasts in response to Bt_2cAMP.

3.3. Secretion of T-kininogen by fibroblasts in response to forskolin, PGE_2, bradykinin and cytokines

We determined whether the exogenous Bt_2cAMP could be replaced by agents that increase intracellular cAMP levels of fibroblasts, namely the adenylate cyclase activators forskolin and PGE_2. As shown in Fig. 4, forskolin or PGE_2 at concentrations above 10 μM or 3 μM, respectively, enhanced the secretion of T-kininogen by meningeal fibroblasts. The level of T-kininogen in the culture medium of meningeal fibroblasts increased linearly up to 3 days after the addition of PGE_2 and reached a plateau at day 4 (data not shown).

We also studied the effect of bradykinin on the T-kininogen secretion by meningeal fibroblasts, since bradykinin stimulates the synthesis of PGE_2 in fibroblasts, which in turn binds to its own receptor and stimulates cAMP synthesis [26]. The addition of 1–10 μM bradykinin caused a concentration-dependent increase in T-kininogen secretion (Fig. 4).

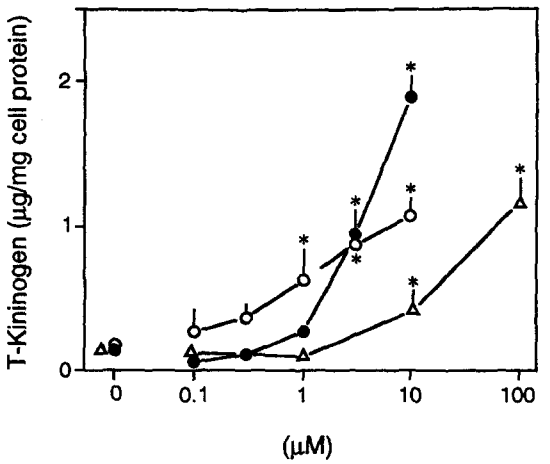

Fig. 4. The effects of forskolin, PGE_2 and bradykinin on the secretion of T-kininogen by rat meningeal fibroblasts. Rat meningeal fibroblasts were cultured for 4 days after the addition of forskolin (△), PGE_2 (●) or bradykinin (○), and the T-kininogen in the medium was assayed by means of a T-kininogen radioimmunoassay. The results are expressed as the means ± S.D. of 4 wells. * Statistical analysis in comparison to unstimulated culture ($P < 0.001$).

Cytokines, such as IL-1 and TNF, are important mediators capable of promoting the inflammatory response at the local and systemic level; they stimulate the synthesis of PGE_2, collagenase production and mitogenesis in fibroblasts, as well as the synthesis of acute-phase proteins in the

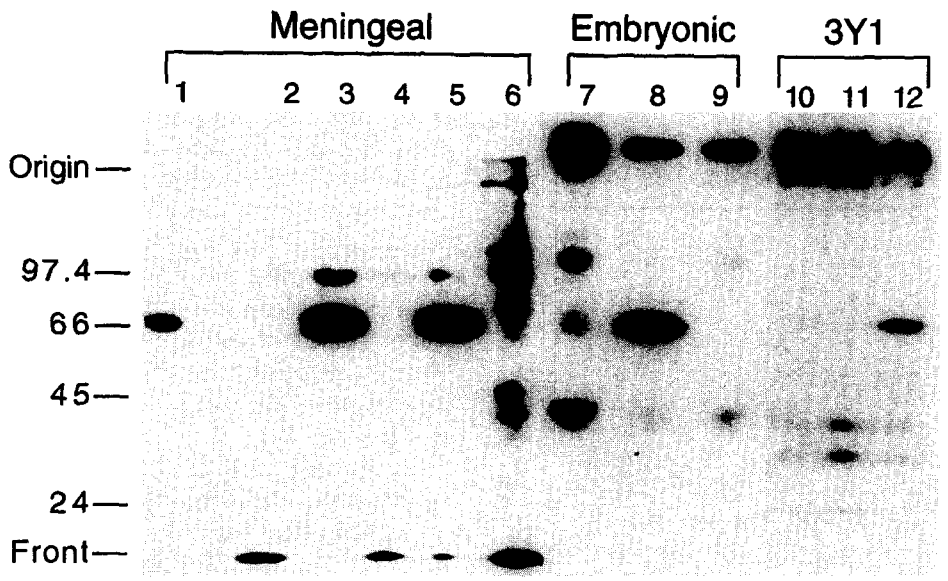

Fig. 3. The incorporation of [^{35}S]methionine into T-kininogen synthesized by rat fibroblasts. Rat meningeal, embryonic and 3Y1 fibroblasts were cultured with or without 1 mM Bt_2cAMP and/or 1 μM dexamethasone for 3 days. The cells were then incubated with medium containing [^{35}S]methionine for 24 h, and the conditioned medium was immunoprecipitated using rabbit anti-T-kininogen antibody (see Section 2). The precipitate was reduced by boiling in 10% 2-mercaptoethanol, then resolved by 10% SDS-PAGE. The photograph is of an autoradiogram of the dried gel. Lane 1, ^{125}I-T-kininogen; lanes 2, 7 and 10, conditioned media of non-treated cells; lanes 3, 8 and 11, conditioned media of cells treated with Bt_2cAMP alone; lane 4, conditioned medium of cells treated with dexamethasone alone; lanes 5 and 12, conditioned media of cells treated with Bt_2cAMP and dexamethasone; lanes 6 and 9, conditioned media of cells treated with Bt_2cAMP were immunoprecipitated in the presence of 20 μg/ml of T-kininogen. The numbers to the left of the gel represent molecular mass standards in kDa.

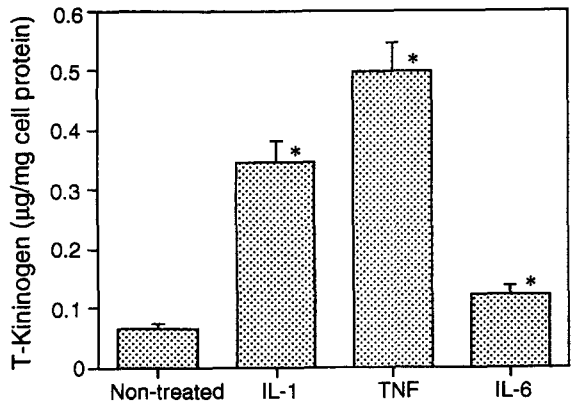

Fig. 5. The effects of cytokines on the secretion of T-kininogen by rat meningeal fibroblasts. Rat meningeal fibroblasts were cultured for 4 days after the addition of IL-1, TNF and IL-6 at a concentration of 500 U/ml, and the T-kininogen in the medium was assay by T-kininogen radioimmunoassay. The results are expressed as the means ± S.D. of 4 wells. * Statistical analysis in comparison to unstimulated culture ($P < 0.001$).

liver [27]. As shown in Fig. 5, both TNF (500 U/ml) and IL-1 (500 U/ml) stimulated T-kininogen secretion by meningeal fibroblasts about 8- and 5-fold, respectively.

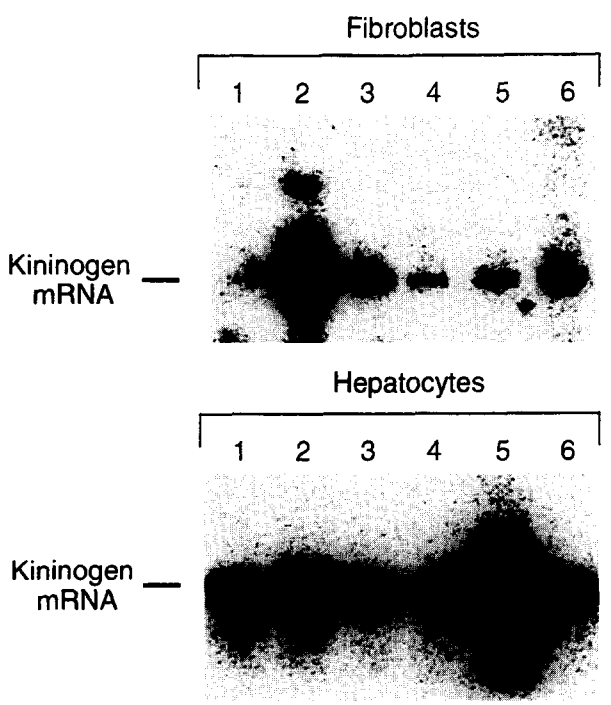

Fig. 6. Representative Northern blots of T-kininogen mRNA in rat meningeal fibroblasts and hepatocytes. Rat meningeal fibroblasts and hepatocytes were cultured for 3 and 2 days, respectively, in the presence or absence of various agents. Total RNA isolated from these cells (20 or 10 μg/lane for fibroblasts or hepatocytes, respectively) was electrophoresed, blotted onto a nylon membrane, and hybridized with T-kininogen cDNA probe. Meningeal fibroblasts (upper panel) and hepatocytes (lower panel): non-treated (lanes 1), exposed to 1 mM Bt_2cAMP (lanes 2), 10 μM PGE_2 (lanes 3), IL-1 (500 U/ml; lanes 4), IL-6 (500 U/ml; lanes 5), and TNF (500 U/ml; lanes 6). * Statistical analysis in comparison to unstimulated culture ($P < 0.001$).

IL-6 (500 U/ml) also stimulated T-kininogen secretion, but to a lesser extent than other cytokines.

3.4. Northern blots of T-kininogen mRNA

Expression of T-kininogen gene by rat fibroblasts was demonstrated by Northern blotting using T-kininogen cDNA as a probe. As shown in the representative autoradiogram in Fig. 6, the message corresponding to the size of T-kininogen mRNA in non-treated fibroblasts was extremely faint. However, the expression of T-kininogen mRNA was dramatically stimulated by 1 mM Bt_2cAMP. The expression was also stimulated by PGE_2 (10 μM), TNF (500 U/ml), IL-1 (500 U/ml), and IL-6 (500 U/ml) (Fig. 6). In contrast to fibroblasts, the expression of T-kininogen mRNA in rat hepatocytes was not altered by these agents, except for IL-6 (500 U/ml), by which the expression of the T-kininogen gene was dramatically stimulated (Fig. 6).

4. Discussion

In this study, we demonstrated for the first time that rat fibroblasts synthesize and secrete T-kininogen. Thus, in addition to hepatocytes, rat fibroblasts are a source of T-kininogen.

We investigated T-kininogen production by rat fibroblasts from three different sources: two primary cultures of fibroblasts from meninges and embryos and one cell line. All of these cells secreted T-kininogen, though at very low levels as compared with that of cultured hepatocytes. However, secretion of T-kininogen by these cells was greatly enhanced by cAMP-elevating agents, such as Bt_2cAMP and forskolin. Similar augmentation by Bt_2cAMP was observed in levels of T-kininogen mRNA, as well as in the incorporation of radioactive methionine into newly synthesized T-kininogen. These results indicated that intracellular cAMP can trigger expression of the T-kininogen gene in fibroblasts. In addition, dexamethasone, which alone had no effect on T-kininogen synthesis, further enhanced the Bt_2cAMP effect on T-kininogen secretion, suggesting that T-kininogen gene expression in fibroblasts is regulated at least by two factors, cAMP and glucocorticoids.

Expression of the T-kininogen gene as well as the secretion of T-kininogen by rat fibroblasts was stimulated by inflammatory mediators and cytokines, such as PGE_2, TNF, IL-1 and IL-6, implying the involvement of T-kininogen expression by fibroblasts in the process of inflammation. In addition to these agents, bradykinin, an active product of the kallikrein-kinin system, stimulated T-kininogen secretion by fibroblasts. PGE_2 is a potent stimulant of cAMP synthesis in fibroblasts [28]. Bradykinin enhances PG synthesis, which, in turn, stimulates cAMP production in fibroblasts [26]. Furthermore, both TNF and

IL-1 stimulate arachidonic acid metabolism in fibroblasts, resulting in the elevation of intracellular cAMP [29,30]. This evidence suggests that the increased concentration of these mediators (except IL-6) in the connective tissues triggers T-kininogen synthesis by fibroblasts through a mechanism that involves cAMP. IL-6 is synthesized and secreted by fibroblasts, and the production is stimulated by cAMP-elevating agents, such as Bt_2cAMP, forskolin, PGE1, TNF and IL-1 [31], suggesting that the increased expression of T-kininogen by these agents is mediated by IL-6 production. However, this possibility can be excluded by the following evidence: IL-6 itself exhibited only a weak stimulatory effect on T-kininogen expression, and the stimulatory effect of Bt_2cAMP was synergistically enhanced by dexamethasone, a potent inhibitor of IL-6 production in fibroblasts [32].

The expression of the T-kininogen gene in either mRNA or protein level was barely detected in cultured rat fibroblasts lacking agents which can increase intracellular cAMP levels. Bt_{2c}AMP or PGE_2 markedly increased the T-kininogen gene expression of fibroblasts, whereas T-kininogen expression by cultured hepatocytes was not affected by Bt_2cAMP, PGE_2, TNF and IL-1, but was greatly stimulated by IL-6, a potent inducer in hepatocytes, of the synthesis of a wide range of acute-phase proteins including T-kininogen [33,34]. Thus, the regulation of T-kininogen expression in fibroblasts is distinct from that in hepatocytes, in terms of the responsiveness to cAMP and inflammatory mediators.

Like other kininogens, T-kininogen is a kinin precursor and a cysteine proteinase inhibitor [2,35]. Plasma and tissue kallikreins do not release kinin from T-kininogen [4]. However, a T-kininogenase, which released the T-kinin from T-kininogen, has been isolated from the rat submandibular gland and identified as a serine protease belonging to the rat tissue kallikrein family [36,37]. T-Kinin is found in plasma as well as in the inflammatory exudate of carrageenin edema in the rat [38], and its pharmacological properties are similar to those of bradykinin [39]. This evidence suggests that T-kininogen acts as a proinflammatory agent at inflammatory sites by releasing T-kinin. On the contrary, T-kininogen, as a cysteine proteinase inhibitor, may enhance wound healing by inhibiting lysosomal proteinases and therefore, play a protective role in tissue injury during inflammation [1]. Unlike other kininogens, T-kininogen is one of several acute-phase proteins whose synthesis by the liver increases during inflammation, leading to a remarkable increase in the plasma T-kininogen concentration which, in turn, may allow an easy access of plasma T-kininogen into inflammatory sites by extravasation. Thus, it has been thought that the source of T-kininogen in damaged tissues is plasma T-kininogen. However, the results presented here raise the possibility that T-kininogen is locally produced and secreted by the connective tissues in response to inflammatory mediators and that it may play a role in the inflammatory processes of local tissues. The precise roles of T-kininogen derived from fibroblasts remain to be determined.

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